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(54) Title: GENE THERAPY USING HOMOLOGOUS RECOMBINATION

(57) Abstract

The present invention is related to a method to correct the mutation(s) behind a genetic disease in the genome of somatic cells derived from an individual afflicted with this disease by contacting said cells with the corresponding non-mutant DNA-fragment to allow it to undergo homologous recombination with and, thus, replace a DNA sequence of the somatic cell genome, wherein said mutation(s) is(are) located. The cells obtained according to the invention can be administered to an individual as a treatment of the said disease. The present invention is also related to a DNA-liposome suspension comprising the said non-mutant DNA-fragment. The said suspension can be used as a DNA-vehicle in the above process. In addition, it can be administered to an individual to obtain correction of mutation(s) by *in vivo* integration of the said DNA into a mutated gene by homologous recombination.

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GENE THERAPY USING HOMOLOGOUS RECOMBINATION

The present invention is generally related to gene therapy as a means for treatment of genetic diseases. More specifically, the invention is related to correction of gene mutation(s) occurring in a gene of an individual and being responsible for a genetic disease.

Although, in a broad sense the expression gene therapy is concerned with the use of genetically modified cells as a system for long-term production and delivery of a desired protein having a therapeutic activity in an individual, the present invention is concerned with gene therapy in a more restricted and commonly adopted sense, viz. the modification of an individual's gene(s) in order to treat a genetic disease.

In principal, gene therapy could be applied to germ cells. However, since such therapy would not be appropriate for the treatment of humans based on ethical as well as practical criteria, the present invention is merely concerned with somatic cell gene therapy.

The primary strategies for somatic cell gene therapy include the deployment of the following general steps: removing cells from an individual to be treated, culturing the cells in vitro, transfecting the cells with foreign DNA, such as a complete gene encoding a desired substance, usually a therapeutic protein, whereafter transfected cells usually are selected and reintroduced into said individual.

These individual steps can be carried out in different ways. For instance, transfection of the cells with foreign DNA is often accomplished with the aid of a vector which carries the DNA into the cell. Presently available approaches to gene therapy make use of infectious vectors, such as retroviral vectors. However, use of an infectious, e.g. retroviral, vector is associated with disadvantages, i.a. an increased risk of occurrence of a tumorigenic insertional event, possibly leading to tumorgenesis; the potential of generating replication-competent viruses during vector production; and short-lived in vivo expression of the product of interest.

The use of vectors to carry the DNA into the cell inherently would introduce into the cell exogenous DNA originating from the vector. However, in therapy it is appreciated if introduction of exogenous DNA other than the exogenous DNA encoding the desired gene product could be avoided.

The present invention is concerned with gene therapy on individuals afflicted with a genetic disease caused by mutation(s), said mutation(s) involving one or a few nucleosides, which is(are) deleted, inserted or substituted, in the gene behind said disease.

More specifically, the present invention is related to a method to correct such

mutation(s) in the genome of somatic cells derived from an individual being in need of such therapy by introduction of an exogenous DNA sequence comprising the corresponding normal, i.e. non-mutant, DNA-fragment into said cell and causing said DNA sequence to undergo homologous recombination with and, thus, replace a DNA sequence of the somatic cell genome, wherein the mutant gene is located.

It is appreciated that according to the present invention introduction of the exogenous DNA sequence and stable integration thereof in the host cell genome can be achieved without the need of an infectious vector, such as a retroviral vector. For instance, introduction of said DNA into the host cells can be accomplished by conventional liposome fusion technique, a liposome suspension being used as DNA vehicle.

Besides, a variety of other techniques are known for introduction of exogenous DNA into recipient cells without the aid of an infectious vector, e.g. electroporation, microinjection, (modified) calcium phosphate or polybrene precipitation and receptor-mediated DNA delivery, which also could be used in accordance with the present invention.

WO 93/09222 is related to gene therapy, wherein somatic cells of vertebrate, particularly mammalian, origin is transfected with exogenous DNA, said exogenous DNA encoding, or being itself, a desired product. Although transfection of host cells including DNA targeting by homologous recombination is disclosed, no clear description of stable integration of exogenous DNA comprising a complete gene into the cell recipient genome is contained therein. Knock-out, replacement or repair of a resident DNA sequence, e.g. an entire gene, is vaguely indicated but not experimentally supported.

It is previously known to use gene knock-out to produce transgenic mice by introducing subtle mutation(s) into the wild type allele of the targeting gene by homologous recombination (Mansour, S.L., Thomas, K.R., Deng, C.X., and Capecchi, M.R. (1990), "Introduction of a lacZ reporter gene into the mouse int-2 locus by homologous recombination", Proc. Natl. Acad. Sci. USA 87:7688-7692).

WO 91/19796 is directed to methods using homologous recombination for modifying endogenous genes in a chimeric or transgenic animal or plant. Methods are disclosed therein which permit production of animal or plant cells, which contain a desired gene which has been inserted into a predetermined gene sequence by homologous recombination. Animal and plant cells can be produced which have subtle and precise modifications of a gene sequence and expression thereof. The methods disclosed therein can be used to produce chimeric or transgenic animals or plants having defined, and specific,

gene alterations, suitably mutations in medically or clinically significant heterologous genes. Although, it is stated therein that the disclosed methods could be used to replace a genetic lesion causing a disease, with a gene sequence encoding a preferred gene product there is no experimental support for this allegation. Furthermore, the efficiency of gene knock-out is low (Jasin M. et al, Genes Devel. 2, 1353-1363 (1988)) and, thus, such methods would be expected to have limited clinical use.

According to the present invention it has unexpectedly been found that it is possible to provide a method for treatment of genetic diseases caused by gene mutation(s) by correcting such mutation(s) by "mutation knock-out", which method is convenient, efficient and well suited for adaption to routine use.

"Mutation knock-out" is used herein to define a type of gene knock-out wherein a mutant sequence is knocked out, i.e. replaced, by a corresponding non-mutant sequence, in order to "repair", i.e. correct, the mutant sequence. Previously, the opposite has been done (Jasin, M. et al., loc. cit.).

According to the present invention replacement of the endogenous DNA sequence is accomplished by homologous recombination. This is a well known natural cellular process, by which cells can transfer a DNA sequence from one DNA molecule to another DNA-molecule, provided that these molecules possess a region of homology with respect to one another, between which regions the DNA transfer may occur. (For further details, see Watson, J.D. in Molecular Biology of the Gene, 3rd Ed., W.A. Benjamin, Inc., Menlo Park, CA (1977)).

Cells, that could be repaired according to the present invention, are all somatic cells, which can be cultured and from which stable cell lines can be established, e.g. lymphocytes, hematopoietic cells, endothelial cells, fibroblasts, myoblasts, epithelial cells and liver cells. Thus, preferably specialized, differentiated cells are used.

The present invention is generally applicable to treatment of genetic diseases caused by mutation(s) in a specific gene, especially by point mutations, small deletions or small insertions. Often more than one mutation is responsible for the disease. Accordingly, if e.g. two mutations are responsible for a disease and these are located within the same region, the disease can be treated with one single non-mutant DNA fragment. Diseases for which the responsible mutations have been identified and which, thus, could be treated according to the present invention are autosomal and X-linked genetic disorders, such as von Willebrand's disease, sickle-cell anemia, β-thalassaemia, haemophilia A and B, and cystic fibrosis.

A specific embodiment of the present invention is concerned with von Willebrand's disease type III.

More specifically, the present invention is related to a method to correct mutation(s) in a gene of animal or human cells by restoring the normal non-mutant DNA-sequence of the gene in order to obtain cells capable of expressing the normal gene product, said method comprising

a) incubating said cells with an exogenous DNA-molecule comprising the normal, non-mutant DNA-fragment and, optionally, flanking sequences, e.g. intron sequences, on each side, said DNA-molecule having a sufficient length to permit said DNA-molecule to undergo homologous recombination with the gene comprising mutation(s)

b) causing the exogenous DNA-molecule to be introduced into the cells,

c) permitting the introduced DNA-molecule to undergo homologous recombination with the gene comprising mutation(s), cells being produced, wherein a fragment of the said gene comprising the mutation(s) has been replaced by the exogenous DNA-molecule, said cells being able to express the normal gene product; and

d) recovering the cells.

Suitably, the cells are derived from an individual, preferably a human, suffering from a genetic disease, as discussed above, and the cells from step d) are administered to said individual to cure or alleviate the disease.

Although it has been reported in the literature that the efficiency of gene knock-out is low, in accordance with the present invention it has been shown that it is possible to increase the said efficiency by repeated gene delivery. Thus, by repeated (e.g. 10-15 times) feeding of a culture of cells, from an individual to be treated, with the exogenous DNA comprising the normal DNA-fragment the amount of repaired, i.e. non-mutant, cells will increase. By repeated feeding at least about 15 times, an amount of repaired cells of at least 0.5 %, suitably 2-5 % or more, of total cells can be achieved.

Since the "repaired" cells obtained according to the present invention virtually are normal somatic cells they will express the normal protein forever (as long as the cells survive) and the expression thereof will be regulated as in normal cells. Thus, if such cells are injected back into the individual to be treated, they can be expected to express the functional protein, i.e. the normal cell product,. to increase, at least to some extent, the concentration thereof in said individual.

The exogenous DNA, which is introduced into the cells and stably integrated into their genome, is preferably comprised of a non-mutant DNA fragment covering at least

the entire exon, which is intended to be repaired in order to restore the function of the gene comprising said exon. Suitably said fragment also covers flanking intron sequences on both side of the exon to facilitate targeting of the homologous recombination. Such fragments can be obtained from cells from individuals not carrying the mutation(s), amplified and cloned by conventional methods. If the DNA sequence has been determined, it is possible, although less convenient, especially for long sequences, to chemically synthesize the desired DNA fragment.

Although the said exogenous DNA usually is comprised of a fragment of a gene, the present invention is not limited to the use of such DNA but an entire gene may constitute the said DNA. If an entire gene is used as exogenous DNA, preferably it has a short DNA-sequence. Moreover, the DNA-fragment could comprise less than an entire exon provided that the length of said fragment is sufficient to allow said homologous recombination to occur.

Liposomes have been shown to be non-toxic in short- and long-term animal tests (San, H. et al., Hum. Gene Therapy 4, 781-788 (1993) and Stewart, M.J. et al., Hum. Gene Therapy 3, 267-275 (1992)). Accordingly, one aspect of the present invention is concerned with treatment of an individual afflicted with a genetic disease, the mutation(s) behind the disease being known, by administration, e.g. by venous injection, of an exogenous DNA liposome-suspension, wherein the exogenous DNA is the normal non-mutant DNA fragment corresponding to the endogenous DNA fragment carrying the mutation(s), to achieve in vivo integration of the exogenous DNA into cell genomes by homologous recombination and, thus, correction of the mutated gene. Cells comprising the corrected gene will express the normal gene product and, thus, the symptoms of the disease can be expected to be reduced or even eliminated.

The following examples are provided to further illustrate the invention through reference to specific embodiments thereof, which are related to the von Willebrand's disease.

On the accompanying drawings, Figure 1 is a diagrammatical representation of the mechanism through which the mutation knock-out is achieved;

Figure 2 A-D illustrate the efficiency of the mutation knock-out in lymphocytes; and Figure 3 shows the breakpoint sequence for the homologous recombination.

The von Willebrand's disease (vWD) is a congenital bleeding disorder related to abnormalities in the von Willebrand Factor (vWF), a blood factor that plays a key role in platelet adhesion, thrombus formation and coagulation. The vWF has two main hemostatic

functions, viz. as a carrier protein of Factor VIII (FVIII) and as a promotor of platelet interaction with damaged vessel walls. As a carrier of FVIII, vWF protects FVIII, which exerts procoagulant activity in the blood coagulation system. In its capacity as a promoter of platelet interactions, vWF mediates platelet adhesion to damaged vessel walls and platelet aggregation, and, thus, thrombus formation.

A severe form of this disease is vWD type III. Patients suffering from vWD type III have very low or even undetectable plasma levels of vWF due to mutations in the gene coding for vWF. Either homozygous, i.e. similar, mutations or compound heterozygous, i.e. different, mutations (Anvret, M. et al., Hum. Genet. 89, 147-154 (1992), Zhang, Z.P. et al., Genomics 21, 188-193 (1994); and Zhang, Z.P. et al., Hum. Mol. Genet. 1, 767-768 (1992)) have been established for such patients.

In the following examples it is shown that such mutations can be corrected in lymphocytes. Other somatic cells, such as endothelial cells and megakaryocytes, that normally express vWF and are easily accessible from the patient to be treated can also be used in accordance with the present invention.

Example 1.

Lymphocytes from a patient with a homozygous nonsense mutation (R1659X/R1659X) in exon 28 of the vWF gene were used to establish Epstein Barr virus (EBV) transformed lymphocytes in a manner known per se (Elisabeth V. Walls and Dorothy H. Crawford, "Generation of human B Lymphoblastoid cell lines using Epstein-Barr virus", 149-162 in Lymphocytes a practical approach, edited by G.G.B. Klaus, IRL Press Limited, Oxford 1987).

A non-mutant DNA fragment comprised of the whole exon 28 and parts of introns 27 and 28 and corresponding to the mutant region, was amplified from normal individuals, cloned and used to feed a culture of the above mentioned lymphocytes. Transfer of the non-mutant DNA fragment into the lymphocytes was mediated by lipofectamine, a positively charged liposome, which can surround the DNA to form small liposome-DNA suspensions (Felgner, P.L. et al., Proc. Natl. Acad. Sci. USA 84, 7413-7417 (1987)). Every time the tested cells were fed with DNA-liposome suspension (once a week), an aliquot was saved for analysis of the repair efficiency.

In Figure 1, the mechanism behind the correction of the mutant gene region is illustrated, an intron being shown as a line and an exon as an open box. The mutant DNA sequence (R1659X) is designated A and the non-mutant DNA fragment is designated B.

To amplify the non-mutant DNA-fragment the primers

Vi27u (5'-TGTGGGAATATGGAAGTCATTG-3') and

Vi28-1d (5'-GTATCTTGGCAGATGCATGTAGC-3')

were used and the fragment was directly cloned into the pCT™II vector (obtained from Invitrogen Corporation, San Diego, CA, USA). The sequencing of the cloned fragment was shown to be correct (Stewart, M.J. et al, Hum. Gene Therapy 3, 267-275 (1992)). The primers

Ve28-2u (5'-ATGGTTCTGGATGTGGCGTTC-3'),

Vi28-3d (5'-CAGAGGTTACCTTGAAGGCA-3') and

Vi28-2d (5'AGGCAAGTTTCAACCAAGGC-3')

were used in Example 2 to analyze the efficiency of the mutation knock-out.

Example 2.

In this example the efficiency of the cell repair after repeated feeding with the non-mutant DNA-fragment/liposome suspension was analyzed.

a) As shown in Figure 1, the mutant sequence comprises a Dde I restriction site. Accordingly the mutant sequence C<u>T</u>GAG is recognized and cleaved by the restriction enzyme Dde I, whereas the corresponding non-mutant sequence C<u>C</u>GAG is not cleaved by DdeI. This indicates that digestion of DNA fragments with DdeI can be used to check whether or not the mutation R1659X has been corrected. More specifically, as shown in Figure 2A the 943 bp fragment amplified with the primers Ve28-2u and Vi28-3d, is cleaved by Dde I into two fragments of 491 bp and 452 bp in the presence of the mutation, the uncleaved fragment of 943 bp representing the non-mutant sequence. To estimate the accuracy of the efficiency of this method, different ratios of non-mutant DNA/mutant DNA (from lymphocytes with mutation R1659X/R1659X) (1:25, 1:50, 1:100, 1:200, 1:400, 1:800, 1:1600, 1:3200, 1:6400, 1:12800) were used as standard controls. See Figure 2A, wherein the following designations are used: <u>a</u>: 100bp ladder, m: mutant DNA, <u>b to k</u>: 1/25, 1/50, 1/100, 1/200, 1/400, 1/800, 1/1600, 1/3200, 1/6400, 1/12800 (non-mutant/mutant DNA), <u>n</u>: non-mutant DNA.

b) In Figure 2B and 2C the results from incubation of the lymphocytes from Example 1 and the liposome suspension of the non-mutant DNA fragment (Example 1, Figure 1) and subsequent analysis of the genomic DNA are shown. The following designations have been used: <u>a</u>: 100 bp ladder, <u>m</u>: mutant DNA, <u>n</u>: non-mutant DNA, <u>1-14</u>: aliquot of manipulated cells fed with DNA-liposome suspension after 1 to 14 times, respectively.

3-5x10^6 manipulated lymphocytes were washed with Opti-MEM I medium

(Gibco, BRL, Life Technologies). 2.0 μg of non-mutant DNA fragment (shown in Figure 1) was mixed with 30 μl of lipofectamine in 100 μl of Opti-MEM I medium for 40 minutes to form the DNA-liposome suspension. After feeding the washed cells with DNA-liposome mixtures in 1 ml of Opti-MEM I serum-free medium for 8 hours (Figure 2B) or 24 hours (Figure 2C), 2 ml of fresh medium with 20 % foetal calf serum (FCS) was added and regular feeding was performed with medium containing 20 % FCS. Repeated feeding was performed once a week of an aliquot containing 3-5x10^6 manipulated cells. The remaining cells were used to isolate genomic DNA

1 μg of genomic DNA was cleaved with 20 unites of Dde I and used for PCR (Polymerase Chain Reaction) (Mullis, K.B., and Faloone, F.A. (1987), "Specific synthesis of DNA in vitro via a polymerase-catalyzed chain reaction", Methods Enzymol. 155:335-350) amplification with the primers Ve28-2u and Vi28-3d in 100 μl of reaction. The PCR conditions included incubation at 95°C for 4 minutes, and 35 step cycles, i.e. 95°C 1 minute, 60°C 1 minute and 72°C 1.5 minutes, and then a final cycle with extension at 72°C for 10 minutes. 17 μl of the PCR products were cleaved by 20 units of Dde I and separated on a 3 % NuSieve GTG (obtained from FMC Bioproduct, Rockland, ME, USA) agarose gel. As the primer Vi28-3d is located downstream of the non-mutant fragment, contamination with the non-mutant fragment in the genomic DNA will not interfere.

c) In order to confirm that the uncleaved fragment (934 bp) was not due to incomplete activity by the restriction enzyme Dde I, the uncleaved fragment was cut out from the agarose gel and reamplified by PCR with the same primers (Ve28-2u and Vi28-2d). The PCR products (17 μl) were digested with 20 units of Dde I. The results are shown in Figure 2 D wherein the following designations have been used: a: 100 bp ladder, m: mutant DNA, n: non-mutant DNA, b and c: reamplified uncleaved fragment.

The results obtained in Example 2b) and c) are illustrated in Figure 2B-D and Figure 3. As is obvious from Figures 2B and 2C, after incubation of the cell line with the DNA-liposome suspension for one week, analysis of the genomic DNA showed a visible uncleaved fragment of 943 bp. Re-amplification of the uncleaved fragment (943 bp) confirmed that the majority of the fragment was non-mutant DNA and only a small portion was mutant (Figure 2D). Furthermore, sequencing (Sanger, F., Nicklen, S., and Coulson, A.R. (1977), " DNA sequencing with chain-terminating inhibitors", Proc. Natl. Acad. Sci. USA 74:5463-5467) of the uncleaved fragment showed that the sequence of the repaired fragment was identical with that of the non-mutant sequence (Figure 3). The homologous recombination was precise as no breakpoints were detected, except where the

mutation was replaced. This result suggested that the mutation (R1659X) in some tested cells (roughly 1/4000) was replaced by the corresponding normal DNA fragment (Figure 2B, 2C), one or both mutant alleles having been corrected in these cells. In other words, a small population of the cells became heterozygous or homozygous for the non-mutant exon 28. It is evident that the amount of the uncleaved band gradually increased with repeated feeding of the cells with the DNA-liposome suspension (Figure 2B, 2C). After the cells were fed 14 times, the "repaired" cells amounted roughly to 0.5 % of the total tested cells.

Example 3

This example is directed to the correction of a mutation in the vWF gene due to deletion of one single cytosine in exon 18, which in Sweden is the most common mutation in the vWF gene and is present in 50 % of mutant chromosomes. Methods equivalent to those disclosed in Example 1 were used to clone a non-mutant DNA fragment covering the exon 18 of the vWF gene, which non-mutant DNA fragment can be used to correct the said mutation in the same way as in Example 1.

In the above examples endothelial cells from a patient, which cells normally express the vWF, may substitute the lymphocytes. Corrected cells that express the functional vWF protein can then be used in therapy by injection of these cells back into the patient.

Claims

1. A method to correct in vitro, mutation(s) in the genome of somatic cells derived from an individual afflicted with a genetic disease, said mutation(s) occurring in the gene behind the said disease, said method comprising
a) contacting said cells with an exogenous DNA sequence comprising the corresponding normal, i.e. non-mutant, DNA-fragment to introduce said DNA into said cells and
b) causing said DNA sequence to undergo homologous recombination with and, thus, replace a DNA sequence of the somatic cell genome, wherein the said mutant gene is located.

2. The method of claim 1, wherein mutation(s) in a gene of animal or human cells is (are) corrected by restoring the normal non-mutant DNA-sequence of the gene in order to obtain cells capable of expressing the normal gene product, said method comprising
a) incubating said cells with an exogenous DNA-molecule comprising the normal, non-mutant DNA-fragment and, optionally, flanking sequences on each side, said DNA-molecule having a sufficient length to permit said DNA-molecule to undergo homologous recombination with the said gene comprising mutation(s),
b) causing the exogenous DNA-molecule to be introduced into the cells,
c) permitting the introduced DNA-molecule to undergo homologous recombination with the gene comprising mutation(s), cells being produced, wherein a fragment of the said gene comprising the mutation(s) has been replaced by the exogenous DNA-molecule, said cells being able to express the normal gene product; and
d) recovering the cells.

3. The method of claim 1 or 2, wherein the exogenous DNA is introduced into the cells by liposome fusion technique, suitably a liposome suspension being used as the DNA vehicle.

4. The method of any preceding claim, wherein the exogenous DNA is repeatedly, suitably 10-15 times or more, brought into contact with the cells to increase the rate of cells, wherein the said mutation(s) is (are) corrected.

5. The method of any preceding claim, wherein the exogenous DNA originates from cells, which comprise the normal non-mutant gene corresponding to the mutant gene intended to be corrected, preferably of the same species origin as the recipient cells.

6. The method of any preceding claim, wherein the mutation(s) to be corrected is (are) responsible for an autosomal or X-linked genetic disorder, such as von Willebrand's

disease, sickle-cell anemia, β-thalassaemia, haemophilia A and B, or cystic fibrosis.

7. The method of any preceding claim, wherein the cells obtained are intended for subsequent reintroduction into the same individual from which they have been derived.

8. A method to treat a genetic disease by correction of mutation(s) in the genome of somatic cells of an individual afflicted with a genetic disease, said mutation(s) occurring in the gene behind the disease, said method comprising introduction, e.g. by venous injection, of an exogenous DNA-liposome suspension, wherein the exogenous DNA is the normal non-mutant DNA fragment corresponding to the endogenous DNA fragment carrying the mutation(s), to achieve in vivo integration of the exogenous DNA into cell genomes by homologous recombination and, thus, correction of the mutated gene.

9. A DNA-liposome suspension, wherein the DNA comprises a normal non-mutant DNA fragment corresponding to a mutant DNA fragment which mutant DNA fragment is responsible for a genetic disorder.

10. Use of cells, wherein mutation(s) has (have) been corrected in accordance with the method of any of claims 1-6 for treatment of a genetic disorder, e.g. selected from von Willebrand's disease, sickle-cell anemia, β-thalassaemia, haemophilia A and B, and cystic fibrosis.

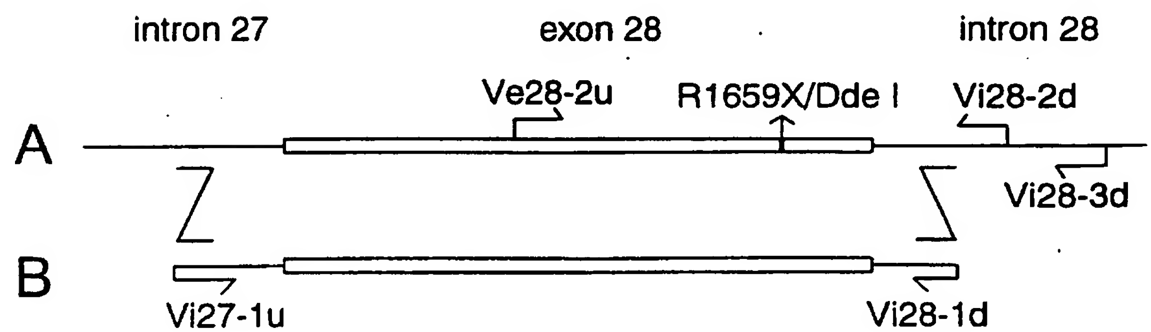
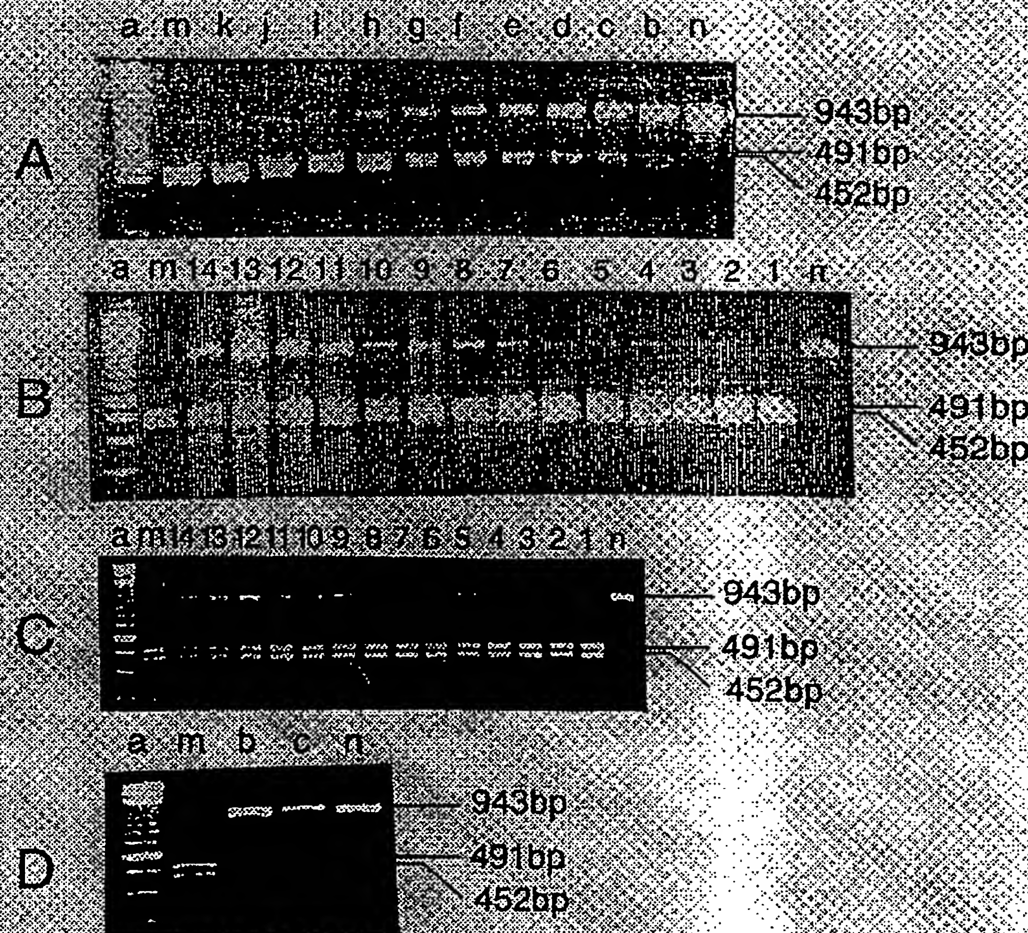

intron 27
exon 28
intron 28
Ve28-2u
R1659X/Dde I
Vi28-2d
A
Vi28-3d
B
Vi27-1u
Vi28-1d

FIG.1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491bp
452bp
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B
943bp
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a m 14 13 12 11 10 9 8 7 6 5 4 3 2 1 n
C
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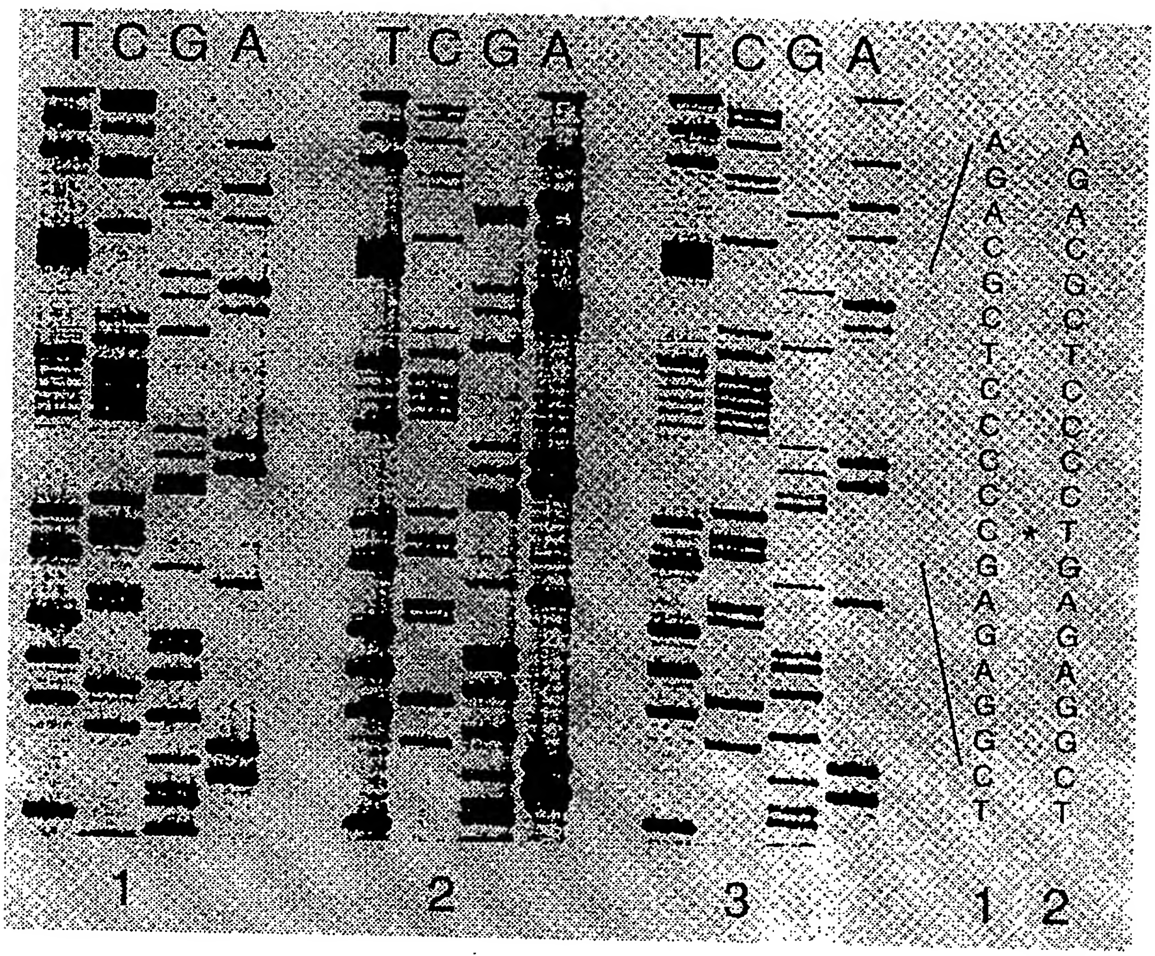
452bp
a m b c n
D
943bp
491bp
452bp

FIG.2

The breakpoint sequence for the homologous recombination.



1: non-mutant sequence. 2: mutant sequence. 3: "repaired sequence".

FIG.3

INTERNATIONAL SEARCH REPORT

International application No.
PCT/SE 94/01038

A. CLASSIFICATION OF SUBJECT MATTER

IPC6: C12N 15/90, C12N 15/00, A61K 48/00, C12N 5/10
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC6: C12N, A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 9404032 A1 (THE REGENTS OF THE UNIVERSITY OF CALIFORNIA), 3 March 1994 (03.03.94), page 12, line 13 - page 13, line 19, example 19, 20, 73 ff. --	1-7,9-10
X	WO 9322443 A1 (SRI INTERNATIONAL), 11 November 1993 (11.11.93), page 10, line 28 - page 11, line 15, example 13, page 51-64 --	1-7,9-10
X	PROC. NATL. ACAD. SCI., Volume 88, May 1991, Edward G. Shesely et al, "Correction of a human betaS-globin gene by gene targeting" page 4294 - page 4298 --	1-2,5,8,10

[X] Further documents are listed in the continuation of Box C. [X] See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" erlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
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"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
4 December 1995	07.12.95.
Name and mailing address of the ISA/ Swedish Patent Office Box 5055, S-102 42 STOCKHOLM Facsimile No. +46 8 666 02 86	Authorized officer Patrick Andersson Telephone No. +46 8 782 25 00

INTERNATIONAL SEARCH REPORT

International application No.
PCT/SE 94/01038

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 9309222 A2 (TRANSKARYOTIC THERAPIES, INC.), 13 May 1993 (13.05.93) --	1-7,9-10
A	WO 9119796 A1 (BAYLOR COLLEGE OF MEDICINE), 26 December 1991 (26.12.91) -- --------	1-7,9-10

INTERNATIONAL SEARCH REPORT

International application No.
PCT/SE94/01038

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.: 8
because they relate to subject matter not required to be searched by this Authority, namely:
See PCT Rule 39.1(iv): Therapeutic treatment of the human or animal body.

2. [ ] Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest
[ ] The additional search fees were accompanied by the applicant's protest.
[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT
Information on patent family members

30/10/95

International application No.
PCT/SE 94/01038

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A1- 9404032	03/03/94	NONE	
WO-A1- 9322443	11/11/93	NONE	
WO-A2- 9309222	13/05/93	AU-A- 3069892	07/06/93
		EP-A- 0649464	26/04/95
		JP-T- 7500969	02/02/95
		PT-A- 101031	28/02/94
WO-A1- 9119796	26/12/91	AU-B- 654284	03/11/94
		AU-A- 8182391	07/01/92
		EP-A,A- 0535144	07/04/93